μ-Crystallin, Thyroid Hormone-binding Protein, is Expressed Abundantly in the Murine Inner Root Sheath Cells

Noriaki Aoki, Kaoru Ito, and Masaaki Ito

Department of Dermatology, Niigata University School of Medicine, Niigata, Japan

In an attempt to investigate the genes expressed during the development of mouse hair follicles, we employed RNA differential display and identified a cDNA encoding μ-crystallin, that is a major component of kangaroo lens and a cytosolic NADP-regulated thyroid hormone-binding protein in human kidney. In northern blot study, *μ-crystallin* transcripts were detected in skin at the highest level among the mouse tissues, whereas lower but detectable in the eye, brain, kidney, heart, lung, and liver. Furthermore, in mouse skin, the gene expression of μ-crystallin followed hair cycle fundamentally, increased significantly during mid- and late anagen phases and decreased during the catagen, telogen, and early anagen phases. *In situ* hybridization revealed that *μ-crystallin* gene starts to be activated in hair cone of anagen III, and that in anagen VI, its expression is detected predominantly in the cuticle layer of the inner root sheath from the upper hair bulb to the middle portion of the keratogenous zone and in the Huxley's layer through the keratogenous zone. The expression was not detected in catagen, telogen, and early anagen hair follicles, and any other skin components. These results suggest the possible involvement of μ-crystallin in the development of mouse hair follicles during the anagen phase. *Key words: hair cycle/RNA differential display. J Invest Dermatol 115:402–405, 2000*

Hair is a keratinized tissue produced under the cycle of growth, regression, and regrowth. This cycle is characterized by three rhythmically interchanging stages. During the telogen phase, the hair follicle remains at rest. The resting dermal papilla is generally in close proximity to the brush-end on the hair. In anagen phase, cell division recommences in a group of epithelial germ cells in contact with the rounded dermal papilla, and a downgrowth of proliferating epithelial cells partly envelopes the dermal papilla. Subsequently, follicle elongation commences and a new hair grows. Within the anagen phase, six stages (anagen I–VI) have been histologically identified (Chase *et al*, 1951). In the catagen phase, hair elongation ceases and the follicle regresses. The mechanisms controlling this cyclic morphogenesis and hair follicle development remain largely unknown despite the identification of numerous regulatory factors that may influence follicle formation (Stenn *et al*, 1996). One possible systematic approach may be to screen genes whose expression levels are controlled during the development and differentiation of the hair follicle.

We employed RNA differential display to identify the differentially expressed genes in the mouse hair follicle during hair growth cycle, and thereby isolated μ-crystallin, a major lens protein in certain marsupial species. In this communication, we describe the gene expression of μ-crystallin in the mouse skin during hair growth cycle.

Manuscript received August 4, 1999; revised January 7, 2000; accepted for publication June 13, 2000.

Reprint requests to: Dr. Noriaki Aoki, Department of Dermatology, Niigata University School of Medicine, Asahimachi-dori, Niigata 951-8510, Japan. Email: rayaoki@med.niigata-u.ac.jp

Abbreviations: IRS, inner root sheath; ORS, outer root sheath; TH, thyroid hormone; TR thyroid hormone receptors.

MATERIALS AND METHODS

Animals and skin samples Syngeneic C57BL/6NCrj female mice (purchased from Charles River, Yokohama, Japan), 1–42 d, and BALB/CAnNCrj mice, 1–42 d, were housed under 12 h light/dark cycles and fed *ad libitum*.

RNA extraction Total RNA were isolated using Isogen (Nippongene, Tokyo, Japan) from the dorsal skin sections that were excised from three C57BL/6NCrj mice ranging in age from day 1 to 42 and the various tissues according to manufacturer's recommendations. All skin samples were examined histologically by a light microscope to confirm the developmental stage in hair growth cycle.

RNA differential display RNA differential display was performed using Delta™ differential display kit according to the manufacturer's protocol (Clontech, Palo Alto, CA). After 10 min digestion with RNase-free DNase (Boehringer, Mannheim, Germany), total RNA samples from dorsal skins of 21 d (telogen phase), 24 d (anagen I), and 27 d (anagen III) C57BL/6NCrj mice were reverse transcribed using the oligo(dT) primer. The cDNA were amplified by polymerase chain reaction (PCR) using one set of the randomly selected arbitrary P primers and oligo(dT) primers, four dNTP including [α-^{33}P]dATP (Amersham Pharmacia Biotech, Bucks, U.K.), and Taq polymerase (Clontech). The PCR products were then separated on 5% denaturing polyacrylamide gels and detected by autoradiography. Differentially amplified fragments were recovered from the gel, and re-amplified under similar conditions. Verified differentially expressed cDNA were subcloned into pGEM-T Easy vector (Promega, Madison, WI), and sequence by automated sequencer (Amersham Pharmacia Biotech). The resulting sequences were used to search for homology with genes registered in GenBank by BLAST.

Northern blot analysis Ten micrograms of total RNA was electrophoresed in 1.5% formaldehyde gel with RNA molecular weight markers and transferred on to a nylon filter as previously described (Thomas, 1983). Additionally, mouse MTN blots (Clontech) to which 2 μg of poly(A)$^+$ RNA was transferred were used according to the manufacturer's instructions for the detailed examination of *μ-crystallin*

mRNA in multiple tissues. Hybridization was performed at 68°C using QuikHyb™ Hybridization Solution (Clontech) and followed by washing with 0.1 × sodium citrate/chloride buffer (150 mM NaCl, 15 mM trisodium citrate), 0.1% sodium dodecyl sulfate at 50°C. The filters were subsequently rehybridized with the ^{32}P-end-labeled oligonucleotide (5′-TGGTCACCA-TGGTAGGCACGGCGACTACCATCGAAAGTTGA-T) derived from 18S rRNA (Raynal *et al*, 1984) or the glyceraldehyde-3-phosphate dehydrogenase probe after removing the *μ-crystallin* signals by boiling in water.

***In situ* hybridization** For the production of the anti-sense and sense RNA probes, *μ-crystallin* cDNA clone covering the partial coding region and 3′ noncoding region was linearized with *Nco*I and *Sal*I, respectively. ^{35}S-UTP-labeled RNA probes were synthesized from the SP6 and T7 promoter with an RNA labeling kit (Amersham Pharmacia Biotech). Similarly, digoxigenin-labeled RNA probes were synthesized using digoxigenin-UTP (Boehringer). I*n situ* hybridization analysis was performed according to the method described previously (Powell and Rogers, 1990). BALB/CAnNCrj mice (21–42 d old) skins were fixed in 4% paraformaldehyde and processes to paraffin sections. The tissue sections hybridized with ^{35}S-labeled probe were dipped into Kodak NTB-2 emulsion, exposed at 4°C for 21 d, and counterstained with hematoxylin. The sections hybridized with digoxigenin-labeled RNA probe were treated with an anti-digoxigenin antibody coupled to alkaline phosphatase and were stained for alkaline phosphatase activity.

RESULTS

Cloning and sequence analysis of the differentially expressed fragments Among several thousand DNA fragments amplified by PCR using 90 combinations of the primers, we identified some cDNA fragments whose band intensity increased during the development from day 21 to day 27 (**Fig 1**). Of them, it was confirmed that clone 7-3AN1 represents part of the open reading frame and 3′ non-coding region of the mouse *μ-crystallin* (accession number AF039391), and this clone was selected for further investigations.

Transcript of *μ-crystallin* is expressed in anagen phase during the hair cycle To clarify the level of the expression of *μ-crystallin* transcript during hair growth cycle, northern blot analysis was performed with the total RNA extracted from the dorsal skin of mice ranging in age from day 1 to 42. This period represents two full cycles of hair growth. The expression of *μ-crystallin* mRNA follows the hair cycle fundamentally, increases during the first anagen phase with its peak at day 13, and is not detectable during catagen and telogen (**Fig 2**). Again, the levels of its mRNA accumulation increase during the anagen phase of the second hair growth cycle. In this second anagen phase, the mRNA accumulation seems to reach a peak at day 36.

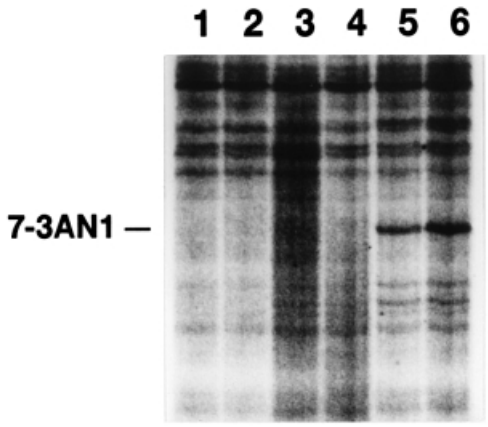

Figure 1. Identification of differentially expressed gene fragments by RNA differential display. Total RNA samples from mouse skin at day 21 (*lanes 1* and *2*), 24 (*lanes 3* and *4*), and 27 (*lanes 5* and *6*) were reverse transcribed and amplified by PCR. The PCR products were analyzed on 5% denaturing polyacrylamide gels. Note that clone 7-2AN1 is detectable only in day 27 (*lanes 5* and *6*).

μ-Crystallin transcript is expressed abundantly in the mouse anagen skin To investigate the expression of μ-crystallin in mouse tissues, northern blot analysis was performed with the total RNA from various mouse tissues. As shown in **Fig 3**(***A***), its transcript was expressed in skin (day 9) at the highest level, preferentially in the eye and brain, and lower but detectable in the kidney. Furthermore, northern blot analysis using poly(A)$^+$ RNA in mouse MTN blots revealed the presence of *μ-crystallin* transcript in the heart, lung, and liver (**Fig 3*B***).

μ-Crystallin* transcript is expressed in the cuticle layer of the inner root sheath (IRS) and Huxley's layer** For *in situ* hybridization, BALB/CAnNCrj mouse was chosen because C57BL/6NCrj mouse from which cDNA clones were isolated had abundant melanin granules in the hair follicles, which interfered with the analysis for the localization of transcripts expressed (Aoki *et al*, 1997). *μ-Crystallin* transcripts were undetected in mouse skin in telogen–anagen II (days 21 and 25) (data not shown), appeared in anagen III–IV (days 26 and 28) (**Fig 4*a–d), expressed abundantly in anagen VI (days 32, 36, and 39) (**Fig 4*e,f***), and again disappeared in catagen phase (day 42) (data not shown). In anagen III when the proliferating epithelial cells form a

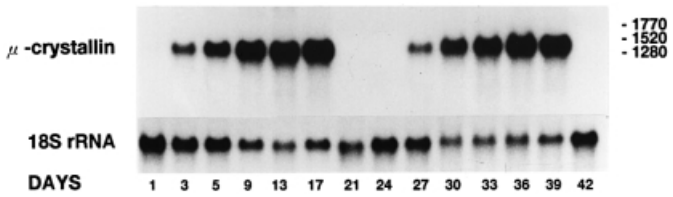

Figure 2. *μ-Crystallin* transcript is expressed specifically in anagen phase during two hair cycles. Total RNA (10 μg) prepared from the dorsal skin of C57BL/6NCrj mouse ranging from day 1 to 42 was electrophoresed and transferred to nylon filter. The filter was hybridized with ^{32}P-labeled *μ-crystallin* cDNA probe and followed by washing with 0.1 × sodium citrate/chloride buffer, 0.1% sodium dodecyl sulfate at 50°C. The blot was subsequently rehybridized with the ^{32}P-end-labeled oligonucleotide derived from 18S rRNA probe.

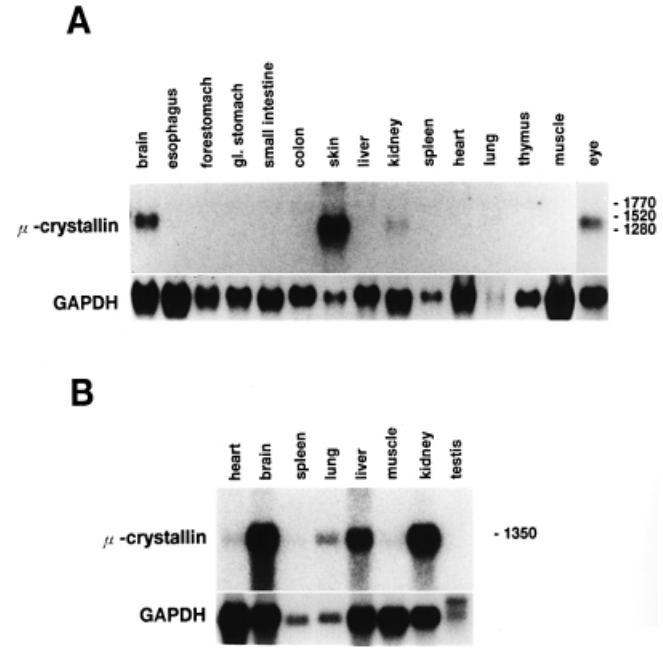

Figure 3. *μ-Crystallin* transcript can be detected abundantly in anagen skin. (*A*) Total RNA was extracted from various tissues of C57BL/6NCrj, separated by electrophoresis in 1.5% agarose, transferred to a nylon filter, and hybridized with ^{32}P-labeled *μ-crystallin* cDNA probe. (*B*) In northern blot using poly(A)$^+$ RNA (2 μg) in mouse MTN blots, *μ-crystallin* is detected in heart, lung, and liver in addition to brain and kidney. RNA sizes are indicated at right.

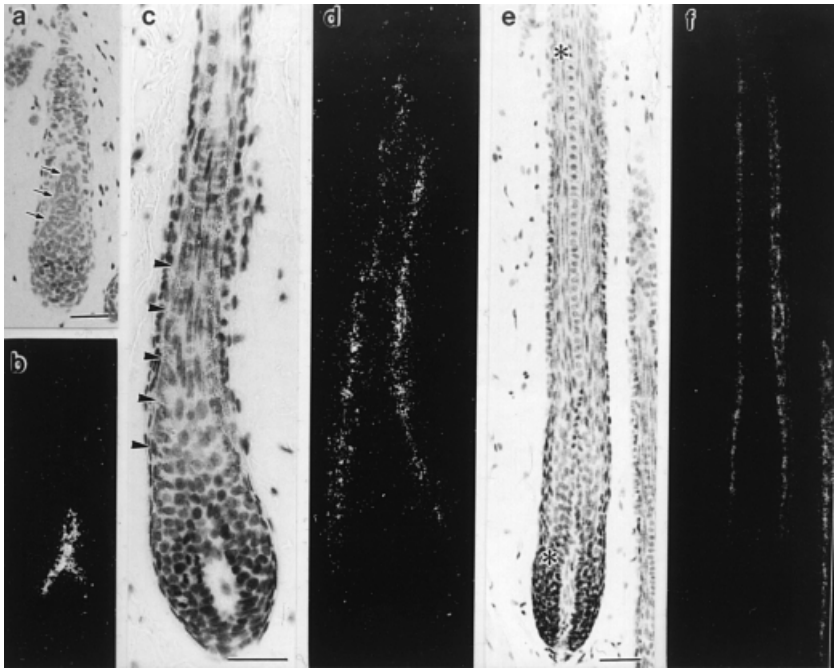

Figure 4. *μ-Crystallin* transcript can be detected in IRS of anagen hair follicle. ^{35}S-UTP-labeled sense and anti-sense RNA probes prepared from mouse *μ-crystallin* cDNA were hybridized to paraffin sections of BALB/CAnNCrj mouse skin at days 26, 28, and 36. (*a*, *c*, *e*) Bright-field views and (*b*, *d*, *f*) darkfield views of longitudinal sections. Note that *μ-crystallin* transcripts appear in hair cone (*arrows*) at day 26 (*a*, *b*) and increase significantly in IRS (*arrowheads*) at day 28 (*c*, *d*). *Asterisks* indicate onset and cessation of *μ-crystallin* mRNA expression in IRS at day 36 (*e*, *f*). No hybridization to the epidermis or any other skin components is seen (data not shown). Sense probe produces random signals (data not shown). *Scale bars*: 50 μm.

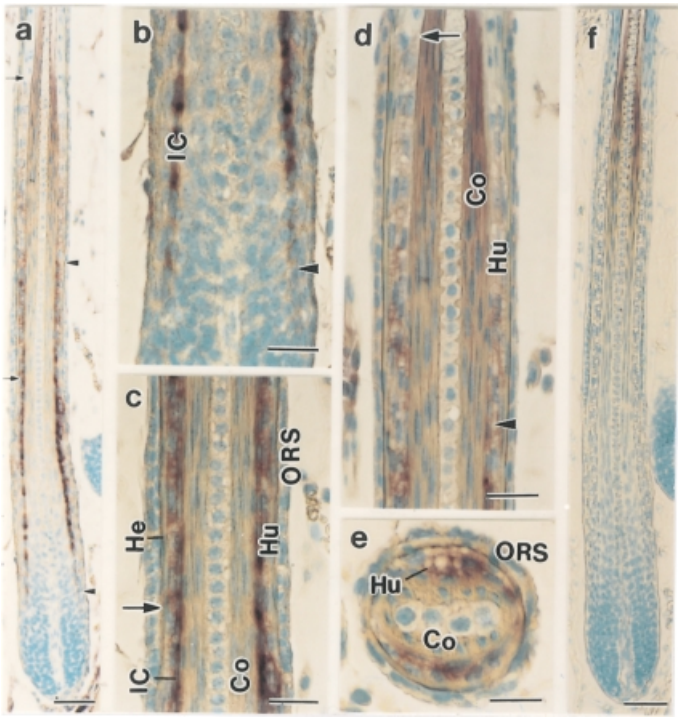

Figure 5. *μ-Crystallin* transcript is expressed in IRS cuticle and Huxley's layers. Digoxigenin-labeled sense and anti-sense RNA probes prepared from *μ-crystallin* cDNA were hybridized to paraffin sections of BALB/CAnNCrj mouse skin at day 36, and counterstained with methyl green (*b–d*). Higher magnifications of the lower, middle, and upper parts of (*a*), respectively. The longitudinal (*a–d*) and transverse (*e*) sections demonstrate that *μ-crystallin* transcripts are detected in IRS cuticles and Huxley's layers. *Arrowheads* and *arrows* indicate onset and cessation of μ-crystallin mRNA expression in IRS cuticle and Huxley's layers, respectively. The occasionally strong staining is seen in the upper cortical layer and dermis, and the similar finding is also in the sections with sense probe (*f*). IC, IRS cuticle; Hu, Huxley's layer; He, Henle's layer; Co, cortex. *Scale bars*: (*a*,*f*) 50 μm; (*b–e*) 25 μm.

follicle bulb around the invaginated dermal papilla and follicle elongation has commenced, signals appeared in the IRS, which is cone shaped inside the outer root sheath (ORS) (**Fig 4*a*,*b***). The stronger signals were detected in the IRS in anagen IV (**Fig 4*c*,*d***). In anagen VI when the follicle has fully elongated, transcripts were predominantly restricted to the IRS, being distributed constantly from the upper hair bulb to the top of the keratogenous zone during this period. *In situ* hybridization results using the digoxigenin-labeled cRNA probe demonstrated the more apparent localization of *μ-crystallin* transcripts in the IRS in anagen VI (**Fig 5**), whereas not in anagens III–IV presumably because of their lower expression. In anagen VI, its transcripts were detected in the IRS cuticle and Huxley's layers. The expression of *μ-crystallin* transcripts in the IRS cuticle layer commenced at the upper hair bulb (**Fig 5*a*,*b***), and was detected until the middle portion of the keratogenous zone (**Fig 5*a*,*d***). In Huxley's layer, its transcripts were expressed throughout the keratogenous zone (**Fig 5*a*,*c*,*d***). Transverse sections confirmed that signals could be detected in IRS cuticle and Huxley's layers but not in the hair shaft and ORS (**Fig 5*e***). During the hair growth cycle, positive signals were not detected in the epidermis, sebaceous gland, and sweat gland (data not shown), whereas were in upper cortical layer (**Fig 5*a*,*d***) and dermis. The sense probe showed the similar positive signals only in the upper cortical layer (**Fig 5*f***) and dermis, indicating that *μ-crystallin* transcripts are not expressed in these skin components.

DISCUSSION

In this study, we demonstrated that *μ-crystallin* mRNA is expressed abundantly in the mouse anagen hair follicle. This was shown by applying differential display reactions to generate cDNA expression patterns of the telogen and early anagen phase, followed by isolation and sequencing of differentially expressed cDNA fragments. μ-Crystallin has been identified as a major lens structural protein in only a few marsupial species (Wistow and Kim, 1991). Outside lens, kangaroo μ-crystallin is preferentially expressed in the retina and brain, presumably in an enzymatic role (Chen *et al*, 1992). In addition, its predicted amino acid sequence shows significant similarities with bacterial ornithine cyclodeaminases and lysine cyclodeaminase, indicating that it is apparently another example of taxon-specific gene recruitment in which an enzyme acquires an additional role as a structural protein (Kim *et al*, 1992; Vié *et al*, 1997) There seems to be a general rule that enzymes recruited as crystallins have a pre-existing function in most lenses

(Wistow *et al*, 1991). Therefore, it has been suggested that μ-crystallin could be an enzyme participating in a process such as osmoregulation essential to maintain lens transparency as an additional role (Kim *et al*, 1992). In humans, μ-crystallin is detectable in the heart, brain, skeletal muscle, and kidney. Our northern blot results demonstrate that similarly preferential expression is detected in the mouse brain and kidney, whereas it is less in the heart and skeletal muscle. The expression of *μ-crystallin* mRNA is confirmed in the eye as well, although it remains to be analyzed whether it is detected in the lens. Interestingly, *μ-crystallin* transcripts are expressed in skin at the highest level among the various mouse tissues.

The IRS is composed of the IRS cuticle, Huxley's, and Henle's layers, which is likely to play a part in guiding the developing cell layers to constitute a hair shaft by forming an inner part of the follicular wall surrounding the hair canal. *In situ* hybridization experiments show that *μ-crystallin* mRNA is expressed abundantly in the IRS cuticle and Huxley's layers during the period from anagen III to anagen VI. This finding indicates that μ-crystallin may be a major structural protein in these mouse hair follicle cells. Furthermore, the notion that μ-crystallin may be involved in osmoregulation in the kangaroo lens raises the interesting possibility that it plays an additional part also in the mouse IRS cuticle and Huxley's cells.

Recently, it has been reported that μ-crystallin corresponds to a major cytosolic NADP-regulated thyroid hormone-binding protein (Vié *et al*, 1997). The major effects of thyroid hormone (hair follicle) occur via the binding to the nuclear TH receptors (TR) that belong to a superfamily of ligand-dependent transcription factors. TR remain bound to their target genes even in the absence of ligand (Lazar, 1993; Parker, 1993), and thus the intracellular transport of TH and the control of its intracellular concentration are undertaken by cytosolic binding proteins (Vié *et al*, 1997). There is clinical and experimental evidence that TH has certain effects on hair growth (Zaun and Perret, 1989a,b). In hypothyroidism, sparse scalp hair, loss of the outer third of the eyebrows, and diminished body hair are often seen, which result from the increase of the relative proportions of telogen compared with anagen hairs and are corrected by the administration of TH, implying that TH affects both the hair cycle and growth of human hair. In fact, TR were detected in the ORS cells, dermal papillae cells, and fibrous sheath cells of human hair follicles; furthermore, *in vitro* studies with the cultured ORS cells and dermal papillae cells indicated that TH might have a direct effect on the growth of hair follicles via TR (Ahsan *et al*, 1998).

In this study, the expression of *μ-crystallin* mRNA is undetected in the ORS, dermal papillae, and fibrous sheath, indicating that the other cytosolic thyroid hormone-binding protein may participate in the intracellular transport of TH in these skin components. Alternatively, *μ-crystallin* mRNA is expressed abundantly in the IRS cuticle and Huxley's cells where the presence of TR has not been detected immunohistochemically (Ahsan *et al*, 1998). Therefore, μ-crystallin, NADP-regulated TH-binding protein, binds TH with high affinity in the cytoplasm of these IRS cells, whereas bound hormone is likely to have no effect on the TR-based development. These observations suggest that this protein may regulate the distribution and concentration of TH in the hair follicle via binding to the hormone within the IRS cuticle and Huxley's cells. In future, the function of μ-crystallin in the hair follicle should be elucidated further by producing transgenic or knockout mice using its gene.

REFERENCES

Ahsan MK, Urano Y, Kato S, Oura H, Arase S: Immunohistochemical localization of thyroid hormone nuclear receptors in human hair follicles and in vitro effect of L-triiodothyronine on cultured cells of hair follicles and skin. *J Med Invest* 44:179–184, 1998

Aoki N, Ito K, Ito M: Isolation and characterization of mouse high-glycine/tyrosine proteins. *J Biol Chem* 272:30512–30518, 1997

Chase HB, Rauch H, Smith VW: Critical stages of hair development and pigmentation in the mouse. *Physiol Zool* 24:1–8, 1951

Chen H, Philips HA, Callen DF, Kim RY, Wistow GJ, Antonarakis SE: Localization of the human gene for μ-crystallin to chromosome 16p. *Genomics* 14:1115–1116, 1992

Forrest D: The erbA/thyroid hormone receptor genes in development of the central nervous system. *Semin Cancer Biol* 5:167–176, 1994

Fraichard A, Chassande O, Plateroti M, *et al*: The T3R α gene encoding a thyroid hormone receptor is essential for post-natal development and thyroid hormone production. *EMBO J* 16:4412–4420, 1997

Freake HC, Oppenheimer JH: Thermogenesis and thyroid function. *Annu Rev Nutr* 15:263–291, 1995

Ito M, Tazawa T, Shimizu N, Ito K, Katsuumi K, Sato Y, Hashimoto K: Cell differentiation in human anagen hair and hair follicles studied with anti-hair keratin monoclonal antibodies. *J Invest Dermatol* 86:563–569, 1986

Kim RY, Gasser R, Wisto GJ: μ-Crystallin is a mammalian homologue of Agrobacterium ornithine cyclodeaminase and is expressed in human retina. *Proc Natl Acad Sci USA* 89:9292–9296, 1992

Koenig RJ, Lazar AA, Hodin RA, Brent GA, Larsen PR, Chin WW, Moore DD: Inhibition of thyroid hormone action by a non-hormone binding T3R protein generated by alternative mRNA splicing. *Nature* 337:659–661, 1989

Lazar MA: Thyroid hormone receptors: multiple forms, multiple possibilities. *Endocr Rev* 14:184–193, 1993

Lazar MA, Hodin RA, Darling DS, Chin WW: Identification of a rat c-erbA alpha-related protein which binds deoxyribonucleic acid but does not bind thyroid hormone. *Mol Endocrinol* 2:893–901, 1988

Mitsuhashi T, Tennyson G, Nikodem V: Nucleotide sequence of novel cDNAs generated by alternative splicing of a rat thyroid hormone receptor gene transcript. *Nucleic Acids Res* 24:5697, 1988

Parker MG: Steroid and related receptors. *Curr Opin Cell Biol* 5:499–504, 1993

Powell BC, Rogers GE: Cyclic hair-loss and regrowth in transgenic mice overexpressing an intermediate filament gene. *EMBO J* 9:1485–1493, 1990

Raynal F, Michot B, Bachellerie JP: Complete nucleotide sequence of mouse 18S rRNA gene: comparison with other available homologs. *FEBS Lett* 167:263–268, 1984

Silva JE: Thyroid hormone control of thermogenesis and energy balance. *Thyroid* 5:481–492, 1995

Thomas PS: Hybridization of denatured RNA transferred or dotted onto nitrocellulose paper. *Methods Enzymol* 100:255–266, 1983

Törmä H, Rollman O, Vahlquist A: Detection of mRNA transcripts for retinoic acid, vitamin D3, and thyroid hormone (c-erb-A) nuclear receptors in human skin using reverse transcription and polymerase chain reaction. *Acta Derm Venereol (Stockh)* 73:102–107, 1993

Vié MP, Blanchet P, Samson M, Francon J, Blondeau JP: High affinity thyroid hormone-binding protein in human kidney; kinetic characterization and identification by photoaffinity labeling. *Endocrinology* 137:4563–4570, 1996

Vié MP, Evrard C, Osty J, *et al*: Functional expression of the human nicodinamide-adenine dinucleotide phosphate-regulated thyroid hormone-binding protein. *Mol Endocrinol* 11:1728–1736, 1997

Wisto G, Kim H: Lens protein expression in mammals: taxon-specificity and the recruitment of crystallins. *J Mol Evol* 32:262–269, 991

Zaun H, Perret C: Internal diseases affecting hair growth. In: Orfanos CE, Happle R (eds.). *Hair and Hair Diseases*. Berlin: Springer-Verlag, 1989a, pp. 267–299

Zaun H, Perret C: Internal diseases affecting hair growth. In: Orfanos CE, Happle R (eds.). *Hair and Hair Diseases*. Berlin: Springer-Verlag, 1989b, pp. 587–600